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## Presence of Human T-Cell Responses to the *Mycobacterium leprae* 45-Kilodalton Antigen Reflects Infection with or Exposure to *M. leprae*

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**The ability of the 45-kDa serine-rich *Mycobacterium leprae* antigen to stimulate peripheral blood mononuclear cell (PBMC) proliferation and gamma interferon (IFN- $\gamma$ ) production was measured in leprosy patients, household contacts, and healthy controls from areas of endemicity in Mexico. Almost all the tuberculoid leprosy patients gave strong PBMC proliferation responses to the *M. leprae* 45-kDa antigen (92.8%;  $n = 14$ ). Responses were lower in lepromatous leprosy patients (60.6%;  $n = 34$ ), but some responses to the 45-kDa antigen were detected in patients unresponsive to *M. leprae* sonicate. The proportion of positive responses to the *M. leprae* 45-kDa antigen was much higher in leprosy contacts (88%;  $n = 17$ ) than in controls from areas of endemicity (10%;  $n = 20$ ). None of 15 patients with pulmonary tuberculosis gave a positive proliferation response to the 45-kDa antigen. The 45-kDa antigen induced IFN- $\gamma$  secretion similar to that induced by the native *Mycobacterium tuberculosis* 30/31-kDa antigen in tuberculoid leprosy patients and higher responses than those induced by the other recombinant antigens (*M. leprae* 10- and 65-kDa antigens, thioredoxin, and thioredoxin reductase); in patients with pulmonary tuberculosis it induced lower IFN- $\gamma$  secretion than the other recombinant antigens. These results suggest that the *M. leprae* 45-kDa antigen is a potent T-cell antigen which is *M. leprae* specific in these Mexican donors. This antigen may therefore have diagnostic potential as a new skin test reagent or as an antigen in a simple whole-blood cytokine test.**

Leprosy is a chronic infectious disease of the skin and peripheral nerves caused by infection with *Mycobacterium leprae*. Leprosy patients present with a spectrum of clinical disease that is closely correlated with the ability of the host to make a cellular immune response to the organism (19). Tuberculoid leprosy patients have strong cell-mediated immunity to *M. leprae* both in vitro and in vivo, and few bacilli are present in skin lesions. At the other end of the spectrum, T cells from lepromatous leprosy patients are nonresponsive to *M. leprae* and there is uncontrolled growth of the organism in skin lesions, often leading to disability and social isolation. The widespread implementation of multidrug therapy during the past 10 years has resulted in a dramatic reduction in the numbers of registered leprosy patients (25). Despite these achievements, it is presently unclear whether the implementation of multidrug therapy has had any effect on *M. leprae* transmission or reduced the incidence of disease (34), as the number of new cases being reported each year has remained the same at over 500,000 worldwide (25). Therefore, a major priority in leprosy research

is the identification of new specific *M. leprae* antigens for use as skin test reagents to identify those who have been exposed to the organism and also to help detect individuals with early subclinical infection (21).

A number of antigens have been reported to induce T-cell responses from tuberculoid leprosy patients and leprosy patient contacts in vitro, including the 70-, 65-, 18-, and 10-kDa heat shock proteins (2, 6, 9, 15, 16, 22, 26), the 30/31-kDa secretory proteins (13–15), and the *M. leprae* 35-kDa antigen (28). However, all these *M. leprae* antigens have been shown to be cross-reactive, with homologous genes identified in other mycobacterial species (27, 36), and thus would not be useful as diagnostic reagents.

The importance of gamma interferon (IFN- $\gamma$ ) in reducing infection with *M. leprae* is well documented in vitro and in vivo. Th1 T cells specific for mycobacterial antigens have been isolated from the lesions and peripheral blood of tuberculoid leprosy patients (8, 17, 23). The skin lesions from tuberculoid leprosy patients have also been shown to contain abundant Th1 cytokine mRNA, which was rare in lepromatous lesions (35). Furthermore, when skin lesions of lepromatous leprosy patients were inoculated with recombinant IFN- $\gamma$ , marked reductions in bacterial load were observed (11). The ability to induce secretion of IFN- $\gamma$  is therefore an important property of those leprosy antigens involved in protective immunity.

Vega-Lopez et al. (29) used pooled sera from lepromatous

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leprosy patients to screen an *M. leprae* lambda gt11 expression library (37) and isolated and sequenced a gene encoding a serine-rich protein with a predicted molecular mass of 45 kDa; (25L or Sra) (27). A high proportion of leprosy patient sera (78% of multibacillary and 68% of paucibacillary leprosy patient sera) contained immunoglobulin G (IgG) antibodies to a  $\beta$ -galactosidase 45-kDa fusion protein (29). Work by others (20) suggested that this antigen may be specific to *M. leprae* as DNA from *Mycobacterium tuberculosis* and several atypical mycobacteria failed to hybridize with a 45-kDa protein-encoding DNA probe in Southern blots. We have now compared peripheral blood mononuclear cell (PBMC) proliferation and IFN- $\gamma$  production by leprosy patients in response to the 45-kDa protein with those induced by other proteins including the 65-, 30/31-, and 10-kDa antigens, as well as the *M. leprae* thioredoxin (Trx) and thioredoxin reductase (TR) proteins (31–32). These responses were compared with those of leprosy contacts, healthy individuals living in the same house as a leprosy patient, patients with pulmonary tuberculosis, and individuals living in a leprosy-endemic area in order to evaluate whether the 45-kDa antigen contains *M. leprae*-specific T-cell epitopes.

#### MATERIALS AND METHODS

**Patient population and controls.** Leprosy patients were recruited from the Centro Dermatológico Dr Ladislao de la Pascua, Mexico City, Mexico, the Hospital del Especialidades de Centro Médico Nacional Siglo XXI (IMSS), Mexico City, Mexico, the Centro Médico La Raza, Mexico City, Mexico, and the Hospital Amigo del Niño y la Mujer, Celaya, Mexico. Patients were categorized according to clinical diagnosis, histopathology, and bacterial index of skin slit smears. A total of 48 leprosy patients were used for the study. The tuberculoid leprosy patient group consisted of 9 polar tuberculoid (TT) and 5 borderline tuberculoid (BT) patients, and the lepromatous leprosy patient group was made up of 34 individuals with the diffuse or nodular forms of lepromatous leprosy. All the leprosy patients recruited were receiving chemotherapy at the time of the study. The contact group consisted of 17 healthy individuals living in the same house as a leprosy patient. Of these, 14 were contacts of lepromatous leprosy patients, 2 were contacts of patients with the indeterminate form of disease, and one was a contact of a borderline case. A further 20 healthy blood donors with no known exposure to the disease were recruited from the Hospital Amigo del Niño y la Mujer, in the leprosy-endemic area of Guanajuato, and Centro Dermatológico Dr Ladislao de la Pascua. Eighteen patients with pulmonary tuberculosis were also recruited from the Instituto Nacional de Enfermedades Respiratorias, Mexico City, Mexico.

**Antigens.** Phytohemagglutinin (PHA-P) was supplied by Difco (Detroit, Mich.) and used at a 1:200 dilution. Purified protein derivative (PPD) from *M. tuberculosis* (batch RT48) was obtained from the Statens Serum Institute (Copenhagen, Denmark). Armadillo-derived *M. leprae* sonicate (batch CD235) (prepared as described in the report of the fifth meeting of the Scientific Working Group on the Immunology of Leprosy, World Health Organization [WHO] document TDR/IMM-LEP-SWG [5] 80.3, annex 4, p. 23, 1980) was kindly provided by R. J. W. Rees (National Institute for Medical Research, Mill Hill, United Kingdom). The 30/31-kDa antigen (1) was purified from the culture filtrate of *M. tuberculosis* H37Rv as outlined previously (5). The *M. leprae* 65- and 10-kDa recombinant antigens used in the study were kindly provided by J. van Embden and M. Singh through the WHO Immunology of Mycobacteria antigen bank.

The *M. leprae* 45-kDa antigen was prepared by subcloning the 45-kDa-protein gene (20) into the pTrcHisB vector (Invitrogen BV, Groningen, The Netherlands); the expressed protein was purified under denaturing conditions using a nickel chelate affinity resin (Qiagen, Chatsworth, Calif.) (31), and the purified protein fractions were dialyzed against phosphate-buffered saline (PBS). The recombinant *M. leprae* Trx and TR, as well as a negative histidine control (His) prepared from *Escherichia coli* containing the same pTrcHisB vector without an insert, were prepared and purified as for the 45-kDa antigen, except that the IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) induction step was performed at 22°C rather than 37°C for the Trx and TR proteins. This was followed by

sonication under nondenaturing conditions to avoid the formation of insoluble aggregates (31–32). The purity of the 45-kDa antigen was confirmed on Coomassie blue-stained sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis gels, and visible contamination with *E. coli* proteins was excluded by immunoblotting using a peroxidase-labeled rabbit anti-*E. coli* antiserum (Dakopatts, Glostrup, Denmark). Single batches of each antigen were used throughout the study. All the purified and recombinant mycobacterial antigens were used at final concentrations of 10, 1, and 0.1  $\mu$ g/ml; data shown are for the responses to the antigens used at 10  $\mu$ g/ml, which was shown to be optimal (results not shown). The histidine tag control was used as a negative control for both the histidine sequence and for any contaminating *E. coli* proteins. This control was used at a final dilution of 1 in 100, comparable to that of the test antigen.

**PBMC separation.** Heparinized blood from healthy contacts, controls from areas of endemicity, and patients was diluted with an equal volume of RPMI 1640 and then layered over Histopaque 1077 (Sigma Chemical Co., Poole, Dorset, United Kingdom), followed by centrifugation at  $400 \times g$  for 30 min. The PBMCs were isolated from the plasma-Histopaque interface, washed three times with Hanks' balanced salt solution (Gibco BRL, Paisley, United Kingdom), and suspended in growth medium consisting of RPMI 1640 (Gibco BRL) containing 10% autologous plasma, 100 U of penicillin/ml, 100  $\mu$ g of streptomycin/ml, and 2 mM L-glutamine (Gibco BRL). PBMCs were counted using the trypan blue exclusion method.

**T-cell proliferation assays.** T-cell proliferation assays were performed as previously described (6). Aliquots of  $2 \times 10^5$  PBMCs in 180  $\mu$ l of growth medium were dispensed into each well of 96-well round-bottom microtiter plates (Gibco BRL) containing either mitogen or antigen in triplicate wells. All the mycobacterial antigens were used at a final concentration of 10  $\mu$ g/ml, which had previously been shown to be optimal. Negative-control cultures contained PBMCs in growth medium alone. Plates were incubated for 6 days at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air and then were pulsed with 1  $\mu$ Ci of methyl-[<sup>3</sup>H]thymidine (specific activity, 2 Ci/mmol; Amersham International, Little Chalfont, United Kingdom). Cells were harvested 16 h later onto glass fiber filter discs (Cambridge Technology, Cambridge, Mass.) using a semiautomatic cell harvester (PHD; Cambridge Technology). The discs were transferred into biovials (Beckman, High Wycombe, United Kingdom), and 1.5 ml of scintillation fluid (Cytoscent ES; ICN Biomedicals Ltd., Irvine, Calif.) was added. Incorporation of [<sup>3</sup>H]thymidine was determined using a Beckman LS 880 scintillation counter. Proliferative responses to the antigens were considered positive when the stimulation index (SI; counts per minute in stimulated cultures divided by counts per minute in unstimulated cultures) was greater than 3 and the increase in counts per minute in stimulated cultures minus that in unstimulated cultures was  $>2,500$  cpm; this criterion was used to calculate the percent responders for each antigen.

**Enzyme-linked immunosorbent assay (ELISA) for the detection of IFN- $\gamma$ .** Supernatants from antigen-stimulated PBMCs and controls were stored at –20°C before testing. Immulon IV microtiter plates (Dynatech, Billingshurst, United Kingdom) were coated with 100  $\mu$ l of monoclonal antibody to human IFN- $\gamma$  (MD-1; Gibco BRL) at 2.5  $\mu$ g/ml in 0.1 M carbonate buffer, pH 9.6, and incubated overnight at 4°C. The contents of the wells were discarded, and then the wells were blocked with 2.5% bovine serum albumin (BSA) in PBS, followed by incubation in a humid box for 1 h at 37°C. After three washes with PBS-Tween 20 (0.05%), sample supernatants (100  $\mu$ l) or the IFN- $\gamma$  reference standard (GIF86–04; kindly provided by Hoffman la Roche, Basel, Switzerland) was added and the plate was incubated for 2 h at 37°C; this was followed by a further four washes with PBS-Tween 20. Rabbit polyclonal antibody to human IFN- $\gamma$  (100  $\mu$ l/well; kindly provided by P. Kaye, London School of Hygiene & Tropical Medicine), diluted in PBS-Tween 20 containing 5% human AB serum and 0.25% BSA, was added in order to detect bound IFN- $\gamma$ , and the plate was incubated for another hour at 37°C. The washing stage was repeated as before, and 100  $\mu$ l of peroxidase-labeled goat anti-rabbit IgG (heavy plus light chains) (KPL; Dynatech, Billingshurst, United Kingdom) was added to all wells. After an incubation of 30 min at 37°C, the plate was washed and 100  $\mu$ l of tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added. After 15 min, the color development was read at 490 nm on a Dynatech MR600 microplate reader. The assay was sensitive down to a lower limit of 3 U/ml and had an upper limit of detection of 400 U/ml. The quantity of IFN- $\gamma$  present in the supernatants was determined from a standard curve.

**Statistical analysis.** A nonparametric test (Mann-Whitney U test) was used to evaluate the statistical significance of the data.

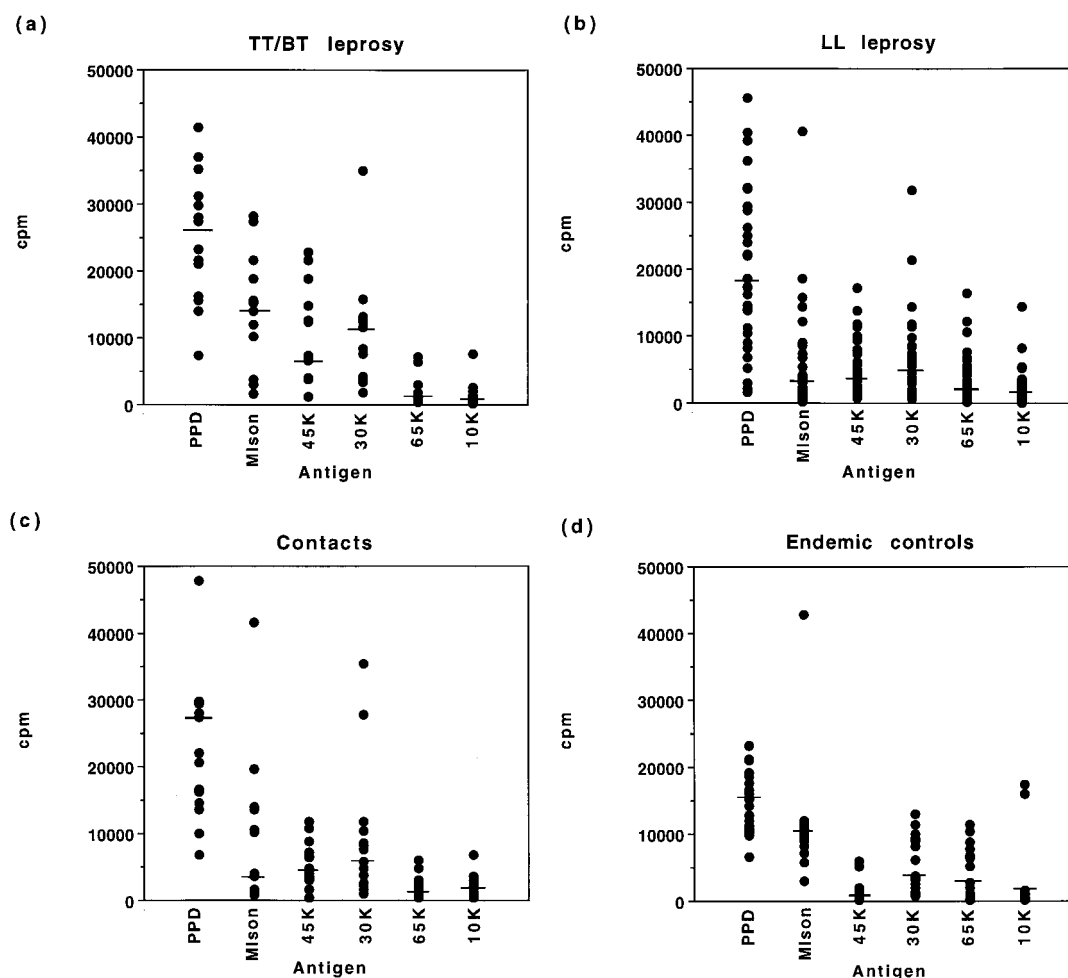


FIG. 1. Lymphocyte proliferation responses induced by a panel of mycobacterial antigens. PBMCs from tuberculoid leprosy patients (TT/BT;  $n = 14$ ) (a), lepromatous leprosy patients (LL;  $n = 34$ ) (b), controls from areas of endemicity ( $n = 20$ ) (c), and leprosy contacts ( $n = 17$ ) (d) were stimulated with a panel of mycobacterial antigens at a final concentration of  $10 \mu\text{g/ml}$  for 6 days as described in Materials and Methods, and proliferation was assessed by uptake of tritiated thymidine. The results are expressed as counts per minute; the median background counts per minute in unstimulated cultures were 126 cpm for TT/BT leprosy patients, 285 cpm for LL leprosy patients, 166 cpm for controls, and 236 cpm for leprosy contacts. The antigens used were PPD of *M. tuberculosis*, *M. leprae* sonicate (Mison), recombinant *M. leprae* 45-kDa antigen (45K), native *M. tuberculosis* 30/31-kDa antigen (30K), recombinant *M. leprae* 65-kDa antigen (65K), and recombinant *M. leprae* 10-kDa antigen (10K). Lines, medians of the groups. Responses were taken as positive if the SI (counts per minute in cultures plus antigen divided by background counts per minute in cultures without antigen) was  $\geq 3$  with an increase in counts per minute (counts per minute in cultures plus antigen – counts per minute in cultures without antigen) of  $\geq 2,500$  cpm.

## RESULTS

**Lymphocyte proliferation responses induced by the *M. leprae* 45-kDa antigen.** The *M. leprae* 45-kDa antigen was expressed as a recombinant protein with a leader sequence of six histidine residues followed by a 24-amino-acid linker at the N terminus (31). It was therefore important to exclude the possibility that there was any human T-cell recognition of this leader sequence. PBMCs from a group of 13 Mexican tuberculoid leprosy patients and 20 controls from an area of endemicity were stimulated with the *M. leprae* 45-kDa antigen or the histidine control prepared as described above. The results confirmed that whereas the *M. leprae* 45-kDa antigen induced positive proliferative responses, the histidine control did not. The inability of the leader sequence to induce positive proliferation responses was also confirmed in a group of 11 United

Kingdom donors and in a group of 13 patients with pulmonary tuberculosis (results not shown).

The PBMC proliferative responses of tuberculoid leprosy and lepromatous leprosy patients were compared with those of healthy household contacts and individuals from a leprosy-endemic area in standard 7-day proliferation assays using a panel of mycobacterial antigens including the 45-kDa antigen (Fig. 1). All leprosy patients, household contacts, and controls from areas of endemicity gave strong proliferative responses to the mitogen PHA (data not shown). The majority of tuberculoid leprosy patients had positive proliferative responses to *M. leprae* sonicate and to the 45-kDa antigen (Fig. 1a). When the proportion of responders was calculated using a SI of  $>3$  and an increase in counts per minute of  $>2,500$  cpm to define a positive response, the tuberculoid leprosy patients showed



92.8% positive responders to both *M. leprae* sonicate and the *M. leprae* 45-kDa antigen. A lower proportion responded to the 65-kDa and 10-kDa antigens (29 and 15%, respectively). Tuberculoïd leprosy patients made significantly higher proliferative responses than lepromatous leprosy patients after stimulation with *M. leprae* sonicate, the *M. leprae* 45-kDa antigen, and the *M. tuberculosis* 30/31-kDa antigen ( $P < 0.05$ ) (Fig. 1b). Both tuberculoïd leprosy and lepromatous leprosy patients made equally good responses to PPD and equally poor responses to the 65-kDa and 10-kDa antigens. Only 35% of the lepromatous leprosy patients failed to make PBMC proliferative responses to *M. leprae* sonicate, and a proportion of these made PBMC proliferative responses to the recombinant *M. leprae* 45-kDa antigen (25%) and to the *M. tuberculosis* 30/31-kDa antigen (36%).

Compared with controls from areas of endemicity, tuberculoïd leprosy patients made significantly higher PBMC proliferative responses to PPD, to 45- and 30/31-kDa antigens ( $P < 0.01$  for all antigens), and to *M. leprae* sonicate ( $P < 0.05$ ) (Fig. 1c). Both groups made similarly low proliferative responses to both the 65- and 10-kDa antigens ( $P > 0.05$ ). However, compared with the household contact group, tuberculoïd leprosy patients made significantly higher responses only to *M. leprae* sonicate ( $P < 0.05$ ) and the 45-kDa antigen ( $P < 0.01$ ). Although the PBMC proliferative responses to PPD and to the 30/31-, 65-, and 10-kDa antigens by tuberculoïd leprosy patients were generally higher than those for the contact group, these differences were not statistically significant.

If the *M. leprae* 45-kDa antigen is an immunodominant leprosy antigen, one would predict that household contacts of leprosy patients might show stronger responses than controls from areas of endemicity. All the controls responded to *M. leprae* sonicate, compared with 55% of the leprosy patient contacts (Fig. 1c and d). In response to the 45-kDa antigen, however, 88% of household contacts made positive PBMC proliferative responses compared with 10% of controls. The *M. tuberculosis* 30/31-kDa antigen induced positive responses in 71% of controls and 55% of household contacts compared with 40 and 12%, respectively, who responded to the 65-kDa protein ( $P < 0.05$ ). When the absolute counts-per-minute values were compared, it was found that there was no significant difference between the response to *M. leprae* sonicate or the 30/31-, 65-, and 10-kDa antigens shown by the contacts and that shown by the controls, although the contact group gave significantly higher PBMC proliferative responses to both PPD and the 45-kDa antigen ( $P < 0.01$ ). Therefore the 45-kDa antigen was the only leprosy antigen which gave significantly higher responses in the contact group than in the controls.

**IFN- $\gamma$  production by PBMCs in response to leprosy antigens.** Supernatants were removed from PBMC cultures after 6 days and assayed for IFN- $\gamma$  by ELISA. There was no significant difference in IFN- $\gamma$  production among any of the groups tested in response to the mitogen PHA ( $P > 0.05$ ) (data not shown). The pattern of IFN- $\gamma$  production in response to the antigens tested in all groups of patients and controls paralleled the PBMC proliferative responses (Fig. 2). The tuberculoïd leprosy patient group produced significantly higher IFN- $\gamma$  than lepromatous leprosy patients in response to PPD, *M. leprae* sonicate, and the 45- and 30-kDa antigens ( $P < 0.05$ ) (Fig. 2a and b). Patients in the tuberculoïd leprosy group produced

significantly higher quantities of IFN- $\gamma$  than the contact group in response to PPD ( $P < 0.01$ ), *M. leprae* sonicate ( $P < 0.02$ ), or the 45- ( $P < 0.01$ ), 30- ( $P < 0.02$ ), and 65-kDa ( $P < 0.01$ ) antigens. Interestingly, IFN- $\gamma$  production in response to the 45-kDa antigen by tuberculoïd leprosy patients was higher than those in response to *M. leprae* sonicate and the 65- and 10-kDa antigens and comparable to that in response to the 30/31-kDa antigen. Compared with that by tuberculoïd leprosy patients, IFN- $\gamma$  production by controls was significantly higher in response to PPD ( $P < 0.01$ ) and the 30- ( $P < 0.02$ ) and 65-kDa ( $P < 0.01$ ) antigens but similar to that in response to *M. leprae* sonicate ( $P > 0.05$ ). IFN- $\gamma$  production by PBMC from lepromatous leprosy patients in response to antigen was similar to that of the contact group for all antigens ( $P > 0.05$ ) except for the 65-kDa antigen, which induced higher IFN- $\gamma$  responses in the lepromatous leprosy patient group ( $P < 0.05$ ). Compared with those from the lepromatous leprosy group, PBMCs from controls gave higher IFN- $\gamma$  production in response to all the antigens except the 45-kDa antigen ( $P < 0.05$ ).

The control group produced significantly higher IFN- $\gamma$  in response to *M. leprae* sonicate, PPD, and the 30- and 65-kDa antigens than the contact group ( $P < 0.01$ ) (Fig. 2c and d). In contrast, IFN- $\gamma$  production by PBMC in response to the 45-kDa antigen was found to be higher in the contact group than in the controls, but this difference was not statistically significant ( $P > 0.05$ ).

**T-cell proliferation responses to the *M. leprae* Trx and TR antigens.** PBMCs from the same groups were also stimulated with two other novel *M. leprae* antigens, prepared as histidine fusion proteins and purified using the same procedure as for the 45-kDa antigen. Neither the Trx nor the TR antigen induced any positive proliferation responses in PBMC from controls from areas of endemicity (Table 1). The Trx antigen induced positive responses in 46% of the leprosy contacts (median, 2,715 cpm) and in 23% of the tuberculoïd leprosy patients (median, 1,633 cpm). The TR antigen induced a similar pattern of responses, although the numbers of responders were lower, with 27% responders in the contact group and 15% responders in the tuberculoïd leprosy group. Both antigens induced low responses in the lepromatous leprosy patients. Therefore although all three recombinant antigens seemed to be potentially leprosy specific, the responses to the *M. leprae* 45-kDa antigen were higher than those to the other fusion proteins. These results also confirmed that the responses to the 45-kDa antigen were not directed to the histidine leader sequence.

**Lymphocyte proliferation and IFN- $\gamma$  responses to the 45-kDa antigen in patients with pulmonary tuberculosis.** The *M. leprae* 45-kDa antigen therefore appeared to induce stronger T-cell responses than the other recombinant leprosy antigens tested and appeared to be more strongly recognized by T cells from leprosy patients and contacts than controls. As a further test of specificity, a group of patients with active tuberculosis was also tested for responses to the *M. leprae* 45-kDa antigen. All the patients with pulmonary tuberculosis responded to PHA (data not shown) and PPD. In contrast, none of the patients tested responded to the 45-kDa antigen and only a small number responded to the other recombinant antigens (Fig. 3a). The responses to *M. leprae* sonicate in the patients with tuberculosis were lower than those in the control group.

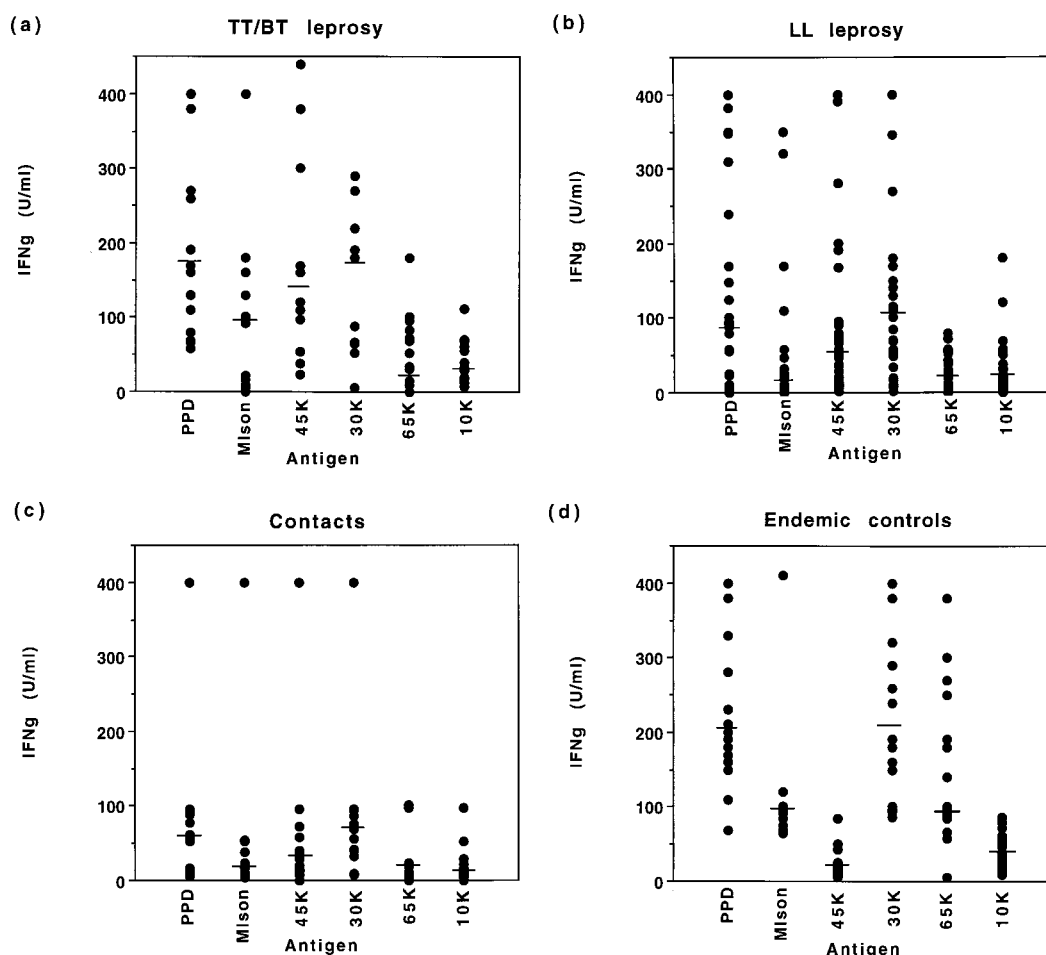


FIG. 2. IFN- $\gamma$  responses induced by a panel of mycobacterial antigens. PBMCs from tuberculoid leprosy patients (TT/BT;  $n = 14$ ) (a), lepromatous leprosy patients (LL;  $n = 34$ ) (b), controls from areas of endemicity ( $n = 20$ ) (c), and leprosy contacts ( $n = 17$ ) (d) were stimulated with a panel of mycobacterial antigens at a final concentration of 10  $\mu\text{g/ml}$  for 6 days as described in Materials and Methods, and IFN- $\gamma$  was measured in the day 6 supernatants by ELISA. The median background concentrations of IFN- $\gamma$  in unstimulated cultures were 3 U/ml for TT/BT leprosy patients, 3 U/ml for LL leprosy patients, 6 U/ml for leprosy contacts, and 4 U/ml for controls. The antigens used were PPD of *M. tuberculosis*, *M. leprae* sonicate (Mlson), recombinant *M. leprae* 45-kDa antigen (45K), native *M. tuberculosis* 30-kDa antigen (30K), recombinant *M. leprae* 65-kDa antigen (65K), and recombinant *M. leprae* 10-kDa antigen (10K). Lines, medians of the groups.

The PBMC proliferative responses to the other antigens tested were generally low and not significantly different from those in the control groups ( $P > 0.05$  for all antigens).

All the pulmonary tuberculosis patients gave strong IFN- $\gamma$  responses to PPD and to the *M. tuberculosis* 30/31-kDa antigen (Fig. 3b). No significant difference in IFN- $\gamma$  production by PBMCs

between tuberculosis patients and controls in response to either PHA or PPD were found ( $P > 0.05$ ). The highest concentrations of IFN- $\gamma$  were produced in response to PPD and to the 65- and 30/31-kDa antigens, although they were not significantly different from those produced by the control group. As seen for the proliferative responses, the *M. leprae* 45-kDa antigen induced lower

TABLE 1. Lymphocyte proliferation responses induced by the *M. leprae* 45-kDa, Trx and TR antigens<sup>a</sup>

Antigen	Response (% responders) for:				
	TT/BT leprosy patients ( $n = 13$ )	LL leprosy patients ( $n = 11$ )	Contacts ( $n = 11$ )	Endemic controls ( $n = 20$ )	Pulmonary tuberculosis patients ( $n = 18$ )
No antigen	126	285	236	166	119
45 kDa	7,398 (92.3%)	3,689 (54.5%)	4,703 (90.9%)	1,030 (10%)	761 (0%)
Trx	1,633 (23.1%)	1,978 (9.1%)	2,715 (45.5%)	529 (0%)	395 (5.6%)
TR	1,326 (15.4%)	1,700 (0%)	1,870 (27.3%)	375 (0%)	375 (5.6%)

<sup>a</sup> PBMC from tuberculoid leprosy (TT/BT) or lepromatous leprosy (LL) patients, leprosy contacts, Mexican controls from regions of endemicity, and patients with pulmonary tuberculosis were cultured with added antigen (no antigen), with Trx or TR antigens (His), with the *M. leprae* 45-kDa antigen, or with *M. leprae* Trx or TR antigens. The results are expressed as median counts per minute as measured by thymidine incorporation as described in Materials and Methods.

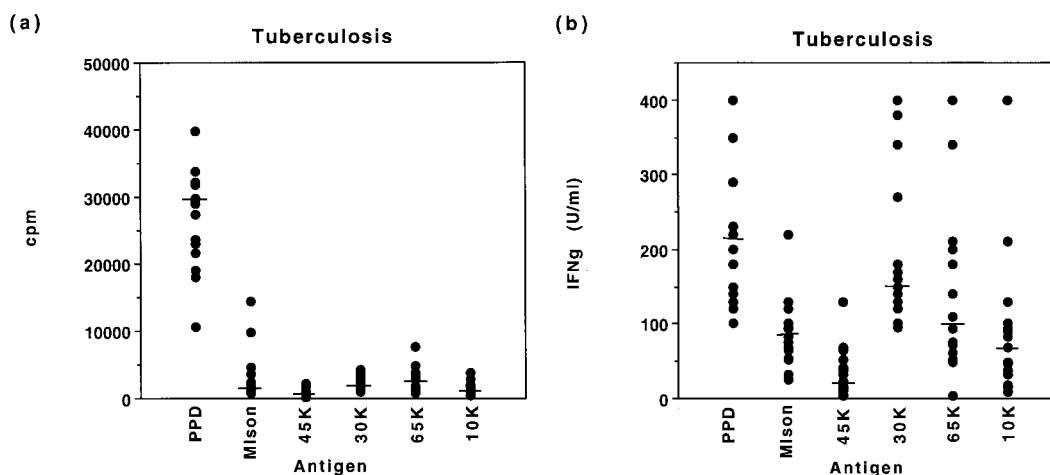


FIG. 3. Lymphocyte proliferation and IFN- $\gamma$  responses in patients with pulmonary tuberculosis. PBMCs from patients with pulmonary tuberculosis ( $n = 18$ ) were stimulated with a panel of mycobacterial antigens at a final concentration of 10  $\mu\text{g/ml}$  for 6 days as described in Materials and Methods, and proliferation was assessed by uptake of tritiated thymidine (a), or IFN- $\gamma$  was measured in the day 6 supernatants by ELISA (b). The median of the background counts in unstimulated cultures was 119 cpm; the median background concentration of IFN- $\gamma$  in unstimulated cultures was 2 U/ml. The antigens used were PPD of *M. tuberculosis*, *M. leprae* sonicate (Mison), recombinant *M. leprae* 45-kDa antigen (45K), native *M. tuberculosis* 30-kDa antigen (30K), recombinant *M. leprae* 65-kDa antigen (65K), and recombinant *M. leprae* 10-kDa antigen (10K). Lines, medians of the groups. For the lymphocyte proliferation assays, responses were taken as positive if the SI (counts per minute in cultures plus antigen divided by background counts per minute in cultures without antigen) was  $\geq 3$  with an increase in counts per minute (counts per minute in cultures plus antigen – counts per minute in cultures without antigen) of  $\geq 2,500$  cpm.

IFN- $\gamma$  responses in the PBMC cultures from patients with pulmonary tuberculosis than any of the other mycobacterial antigens.

A comparison of the percent responders in the lymphocyte proliferation assay to both *M. leprae* sonicate and the *M. leprae* 45-kDa antigen between tuberculosis patients and leprosy patients, contacts, and controls is shown in Fig. 4. This highlights the difference in recognition of the 45-kDa antigen in subjects known to be infected or exposed to *M. leprae* compared to the

patients infected with *M. tuberculosis* and suggests that the *M. leprae* 45-kDa antigen is being recognized as an *M. leprae*-specific antigen.

## DISCUSSION

Multidrug therapy has been highly successful in the treatment of leprosy. However, the identification of novel antigens

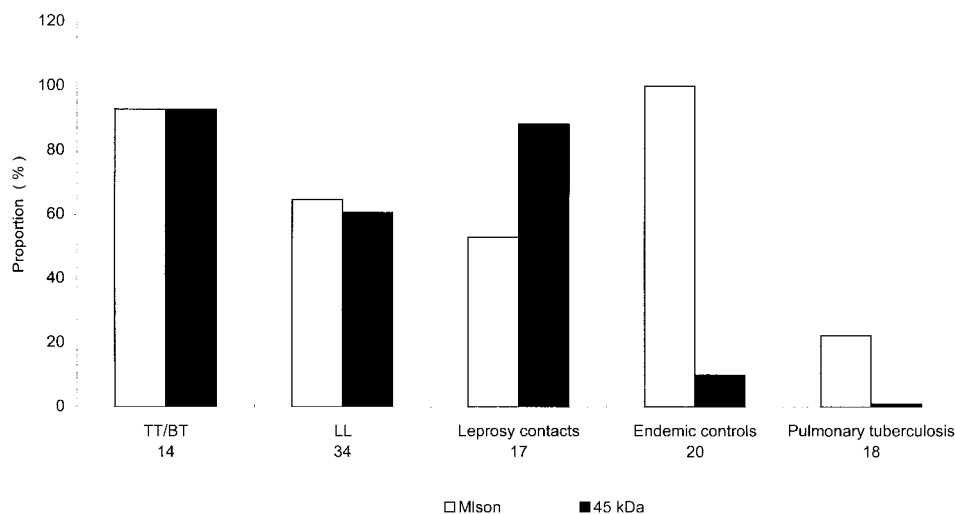


FIG. 4. Comparison of percent responders to *M. leprae* sonicate and *M. leprae* 45-kDa antigen in leprosy patients, leprosy contacts, controls from areas of endemicity, and tuberculosis patients, as measured by lymphocyte proliferation. PBMCs were stimulated with 10  $\mu\text{g}$  of *M. leprae* sonicate (Mison)/ml or *M. leprae* 45-kDa antigen, as described in Materials and Methods, and proliferation was assessed by uptake of tritiated thymidine. A positive response (responder) was defined as that giving a SI (counts per minute in cultures plus antigen divided by background counts per minute in cultures without antigen) of  $\geq 3$  and an increase in counts per minute (counts per minute in cultures plus antigen – counts per minute in cultures without antigen) of  $\geq 2,500$  cpm. The group sizes were 14 for tuberculoid leprosy (TT/BT), 34 for lepromatous leprosy (LL), 17 for leprosy contacts, 20 for controls, and 18 for pulmonary tuberculosis.

which could be used as specific, diagnostic antigens for leprosy is still of major importance. The aims of the present study were to test T-cell reactivity to the 45-kDa antigen, previously described as an *M. leprae*-specific protein (20, 29), and to evaluate the potential of the 45-kDa protein to identify individuals exposed to *M. leprae* or with subclinical infection. A specific skin test reagent could be an important epidemiological tool in monitoring *M. leprae* transmission and disease distribution. The results demonstrate that the *M. leprae* 45-kDa protein is a potent T-cell antigen and that it may have diagnostic potential.

Tuberculoid leprosy patients showed the greatest T-cell responses to the *M. leprae* 45-kDa antigen, and, as 13 of 14 of the tuberculoid leprosy patients tested responded to the 45-kDa antigen, it may contain one or more genetically permissive or promiscuous T-cell epitopes. Some lepromatous leprosy patients also showed good T-cell responses to *M. leprae* and to the *M. leprae* 45-kDa antigen. Although lepromatous leprosy patients are generally anergic to *M. leprae* (10), they can show T-cell responses to individual *M. leprae* antigens (18, 26). The 45-kDa antigen was similar to the secreted *M. tuberculosis* Ag85 (30/31-kDa) proteins in its capacity to stimulate T-cell responses in leprosy patients and contacts. Secreted proteins often appear to be immunodominant in the T-cell response to *M. tuberculosis* (3), and, in leprosy, the 30/31-kDa Ag85 antigen has been shown to induce strong responses in leprosy contacts (15). The high response rates to the 45-kDa antigen reported here are higher than those reported in Ethiopia using the L1 or L4 fragment of the 45-kDa antigen (20). Another study in Indonesia (12) showed that the histidine-tagged *M. leprae* 45-kDa fusion protein only induced positive responses in 3 to 8% of the leprosy patients and contacts tested. Further work is required to assess if the 45-kDa antigen gives stronger T-cell responses in some ethnic groups than others.

T-cell responses to the *M. leprae* Trx and TR antigens were also assessed using recombinant antigens expressed and purified in the same way as the 45-kDa antigen. *M. leprae* is unusual in that these proteins are expressed as a single fusion protein (33). Both of these antigens were recognized by a proportion of leprosy patients and household contacts but not by controls, indicating the presence of *M. leprae*-specific epitopes. Trx and TR could therefore also have potential as diagnostic reagents, although overall the responses induced were less strong than those stimulated by the 45-kDa antigen.

An important aim of this study was to assess the potential of the *M. leprae* 45-kDa antigen as a diagnostic reagent. PBMC proliferative and cytokine responses of patients were compared with those of household contacts of leprosy patients and individuals living in a leprosy-endemic area. The leprosy contact group, generally considered at increased risk of developing leprosy (7), showed very high responses to the *M. leprae* 45-kDa antigen. Follow-up studies would be required to determine whether those contacts making poor T-cell responses to the 45-kDa antigen are at greater risk of developing leprosy, as proposed for poor IFN- $\gamma$  responders to *M. leprae* sonicate (24). Similarly high proportions of contacts have been reported to respond to other leprosy antigens such as the 35-, 18-, and 10-kDa antigens (6, 15, 28) and the *Mycobacterium bovis* BCG 30/31-kDa antigen (15). The *M. leprae* 65-kDa antigen gave lower responses, although the proportions of responders differed in other studies (2, 9, 15).

One of the functions of a skin test reagent is to differentiate individuals exposed to *M. leprae* from those merely living in areas of endemicity. Only 10% of controls responded to the 45-kDa antigen compared with the 100% that responded to *M. leprae* sonicate and 55% that responded to the 30/31-kDa antigen, both of which contain cross-reactive T-cell epitopes. Southern hybridization with a DNA fragment of the 45-kDa protein gene failed to identify a similar gene in *M. tuberculosis* or eight other atypical mycobacteria (20), although several hypothetical protein homologues in *M. tuberculosis* have now been identified (e.g., Rv0442c and Rv2108 [4]).

In conclusion, the data presented here show that the 45-kDa protein is a potent T-cell antigen capable of inducing PBMC proliferation and IFN- $\gamma$  production in leprosy patients. The 45-kDa antigen was strongly recognized by T cells from leprosy patients and contacts but gave weak or negative responses in controls and patients with tuberculosis. This suggests that the *M. leprae* 45-kDa antigen may be of use as a diagnostic reagent, either in a skin test or a simple whole-blood IFN- $\gamma$  assay (30).

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#### REFERENCES

1. Abou-Zeid, C., T. L. Ratliff, H. G. Wiker, M. Harboe, J. Bennedsen, and G. A. Rook. 1988. Characterization of fibronectin-binding antigens released by *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. *Infect. Immun.* 56:3046-3051.
2. Adams, E., R. J. Garsia, L. Hellqvist, P. Holt, and A. Basten. 1990. T cell reactivity to the purified mycobacterial antigens p65 and p70 in leprosy patients and household contacts. *Clin. Exp. Immunol.* 80:206-212.
3. Andersen, P. 1997. Host responses and antigens involved in protective immunity to *Mycobacterium tuberculosis*. *Scand. J. Immunol.* 45:115-131.
4. Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, et al. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393:537-544.
5. De Bruyn, J., K. Huygen, R. Bosmans, M. Fauville, R. Lippens, J.-P. van Vooren, P. Falmagne, M. Weckx, H. G. Wiker, M. Harboe, et al. 1987. Purification, characterisation and identification of a 32kDa protein antigen of *Mycobacterium bovis* BCG. *Microb. Pathog.* 2:351-366.
6. Dockrell, H. M., N. G. Stoker, S. P. Lee, M. J. Jackson, K. A. Grant, N. F. Jouy, S. B. Lucas, R. Hasan, R. Hussain, and K. P. W. J. McAdam. 1989. T-cell recognition of the 18-kilodalton antigen of *Mycobacterium leprae*. *Infect. Immun.* 57:1979-1983.
7. Fine, P. E. M., J. A. C. Sterne, J. M. Ponnighaus, L. Bliss, A. Chihana, M. Munthali, and D. K. Warndorff. 1997. Household and dwelling contact as risk factors for leprosy in northern Malawi. *Am. J. Epidemiol.* 146:91-102.
8. Haanen, J. B., R. de Waal Malefijt, P. C. Res, E. M. Kraakman, T. H. Ottenhoff, R. R. de Vries, and H. Spits. 1991. Selection of a human T helper type-1 like T cell subset by mycobacteria. *J. Exp. Med.* 174:583-592.
9. Ilangumaran, S., N. P. Shanker Narayan, G. Ramu, and V. R. Muthukaruppan. 1994. Cellular and humoral immune responses to recombinant 65-kD antigen of *Mycobacterium leprae* in leprosy patients and healthy controls. *Clin. Exp. Immunol.* 96:79-85.
10. Kaplan, G., D. E. Weinstein, R. M. Steinman, W. R. Levis, U. Elvers, M. E. Patarroyo, and Z. A. Cohn. 1985. An analysis of *in vitro* T cell responsiveness in lepromatous leprosy. *J. Exp. Med.* 162:917-929.
11. Kaplan, G., N. K. Mathur, C. K. Job, I. Nath, and Z. A. Cohn. 1989. Effect of multiple interferon gamma injections on the disposal of *Mycobacterium leprae*. *Proc. Natl. Acad. Sci. USA* 86:8073-8077.
12. Klatzer, P. R., A. M. Janson, J. E. Thole, S. Buhrer, C. Bos, H. Soebono, and R. R. de Vries. 1997. Humoral and cellular immune reactivity to recombinant *M. leprae* antigens in HLA-typed leprosy patients and healthy controls. *Int. J. Lepr. Other Mycobact. Dis.* 65:178-189.
13. Launois, P., K. Huygen, J. De Bruyn, M. N'Diaye, B. Diouf, J. L. Sarthou, J. Grimaud, and J. Millan. 1991. T cell response to purified filtrate antigen 85 from *Mycobacterium bovis* bacille Calmette-Guerin (BCG) in leprosy



- patients. Clin. Exp. Immunol. **86**:286–290.
14. Launois, P., M. Niang N'Diaye, J. De Bruyn, J. L. Sarthou, F. Rivier, A. Drowart, J. P. Van Vooren, J. Millan, and K. Huygen. 1993. T cell stimulation with purified mycobacterial antigens in patients and healthy subjects infected with *Mycobacterium leprae*: secreted antigen 85 is another immunodominant antigen. Scand. J. Immunol. **38**:167–176.
  15. Launois, P., M. Niang N'Diaye, J. L. Cartel, I. Mane, A. Drowart, J. P. Van Vooren, J. L. Sarthou, and K. Huygen. 1995. Fibronectin-binding antigen 85 and the 10-kilodalton Gro-ES related heat shock protein are the predominant Th-1 response inducers in leprosy contacts. Infect. Immun. **63**:88–93.
  16. Mehra, V., B. Bloom, A. C. Bajardi, C. L. Grisso, P. A. Sieling, D. Alland, J. Convit, X. Fan, S. W. Hunter, P. J. Brennan, T. H. Rea, and R. L. Modlin. 1992. A major T cell antigen of *Mycobacterium leprae* is a 10-kD heat-shock cognate protein. J. Exp. Med. **175**:275–284.
  17. Mutis, T., E. M. Kraakman, Y. E. Cornelisse, J. B. A. G. Haanen, H. Spits, R. R. P. De Vries, and T. H. M. Ottenhoff. 1993. Analysis of cytokine production by mycobacterium-reactive T cells. Failure to explain *Mycobacterium leprae*-specific nonresponsiveness of peripheral blood T cells from lepromatous leprosy patients. J. Immunol. **150**:4641–4651.
  18. Ottenhoff, T. H., P. J. Converse, N. Gebre, A. Wondimu, J. P. Ehrenberg, and R. Kiessling. 1989. T cell responses to fractionated *Mycobacterium leprae* antigens in leprosy. The lepromatous nonresponder defect can be overcome in vitro by stimulation with fractionated *M. leprae* components. Eur. J. Immunol. **19**:707–713.
  19. Ridley, D. S., and W. H. Jopling. 1966. Classification of leprosy according to immunity. A five-group system. Int. J. Lepr. Other Mycobact. Dis. **34**:255–273.
  20. Rinke de Wit, T. F., J. E. Clark-Curtiss, F. Abebe, A. H. Kolke, A. A. M. Janson, M. Van Agterveld, and J. E. R. Thole. 1993. A *Mycobacterium leprae*-specific gene encoding an immunologically recognised 45kDa protein. Mol. Microbiol. **10**:829–838.
  21. Roche, P., H. Dockrell, and P. Brennan. 1998. Progress in research towards a world without leprosy. Lepr. Rev. **69**:151–159.
  22. Roche, P. W., W. J. Theuvsen, and W. J. Britton. 1992. Cellular immune responses to Mycobacterial heat shock proteins in Nepali leprosy patients and controls. Int. J. Lepr. **60**:36–43.
  23. Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science **254**:279–281.
  24. Sampaio, E. P., A. L. Moreira, G. Kaplan, M. F. S. Alvim, N. C. Duppre, C. F. Miranda, and E. N. Sarno. 1991. *Mycobacterium leprae*-induced interferon- $\gamma$  production by household contacts of leprosy patients: association with the development of active disease. J. Infect. Dis. **164**:990–993.
  25. Smith, W. C. S. 1997. We need to know what is happening to the incidence of leprosy. Lepr. Rev. **68**:195–201.
  26. Thole, J. E. R., A. A. M. Janson, A. Kifle, R. C. Howe, K. McLean, A. Nurilayn, E. Filey, E. J. Shannon, G. J. Bulla, J. Hermans, R. R. P. de Vries, D. Frommel, and T. F. Rinke de Wit. 1995. Analysis of T-cell and B-cell responses to recombinant *M. leprae* antigens in leprosy patients and in healthy contacts: significant T-cell responses to antigens in *M. leprae* nonresponders. Int. J. Lepr. Other Mycobact. Dis. **63**:369–380.
  27. Thole, J. E. R., B. Wiele, J. E. Clark-Curtiss, T. H. M. Ottenhoff, and T. F. Rinke de Wit. 1995. Immunological and functional characterization of *Mycobacterium leprae* protein antigens: an overview. Mol. Microbiol. **18**:791–800.
  28. Triccas, J. A., P. W. Roche, N. Winter, C. G. Feng, R. C. Butlin, and W. J. Britton. 1996. A 35-kilodalton protein is a major target of the human immune response to *Mycobacterium leprae*. Infect. Immun. **64**:5171–5177.
  29. Vega-Lopez, F., L. A. Brooks, H. M. Dockrell, K. A. L. De Smet, J. K. Thomson, R. Hussain, and N. G. Stoker. 1993. Sequence and immunological characterization of a serine-rich antigen from *Mycobacterium leprae*. Infect. Immun. **61**:2145–2153.
  30. Weir, R. E., A. R. Morgan, W. J. Britton, C. R. Butlin, and H. M. Dockrell. 1994. Development of a whole blood assay to measure T cell responses to leprosy: a new tool for immuno-epidemiological field studies of leprosy immunity. J. Immunol. Methods **176**:93–101.
  31. Wiele, B., S. Nagai, H. G. Wiker, M. Harboe, and T. H. M. Ottenhoff. 1995. Identification and functional characterization of Trx of *Mycobacterium tuberculosis*. Infect. Immun. **63**:4946–4948.
  32. Wiele, B., J. van Noort, J. W. Drijfhout, R. Offringa, A. Holmgren, and T. H. M. Ottenhoff. 1995. Purification and functional analysis of the *Mycobacterium leprae* Trx/TR hybrid protein. J. Biol. Chem. **270**:25604–25606.
  33. Wiele, B., D. van Soolingen, A. Holmgren, R. Offringa, T. T. Ottenhoff, and J. Thole. 1995. Unique gene organisation of Trx and TR in *Mycobacterium leprae*. Mol. Microbiol. **16**:921–929.
  34. World Health Organization. 1998. Trends in leprosy detection. Wkly. Epidemiol. Rec. **73**:169–176.
  35. Yamamura, M., K. Uyemura, R. J. Deans, K. Weinberg, T. H. Rea, B. R. Bloom, and R. L. Modlin. 1991. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. Science **254**:277–279.
  36. Young, D. B., S. H. Kaufmann, P. W. Hermans, and J. E. Thole. 1992. Mycobacterial protein antigens: a compilation. Mol. Microbiol. **6**:133–145.
  37. Young, R. A., V. Mehra, D. Sweetser, T. Buchanan, J. Clark-Curtiss, R. W. Davis, and B. R. Bloom. 1985. Genes for the major protein antigens of the leprosy parasite *Mycobacterium leprae*. Nature **316**:450–452.